DEVELOPMENT OF A NON-INVASIVE OPTICAL IMAGING METHOD FOR TRACKING VASCULAR GENE EXPRESSION

H. H. Chen[1], A. Kumar[2], Y. Yang[2], D. Wang[2], D. Maouyo[3], N. M. Fried[4], X. Yang[2]

[1]Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland, USA

[2]Department of Radiology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[3]Division of Nephrology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[4]Department of Urology, Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

***Abstract*- Gene therapy is an exciting frontier in modern medicine. To date, no imaging modalities are available for monitoring vascular gene therapy. Green fluorescent protein (GFP) has become an increasingly common marker for gene therapy. We have developed an optical imaging method to track vascular gene expression by detecting fluorescence emitted from GFP or red fluorescent protein (RFP) in arterial walls following gene transfer. We surgically transferred GFP- and RFP-vectors into the femoral and carotid arteries of three New Zealand white rabbits. Excitation light was transmitted through a fiber-optic ring-light (Nevoscope) and GFP and RFP fluorescence was detected by a charge coupled device (CCD) camera. Direct contact images of the target arteries demonstrated that this method was capable of both discriminating between normal and transferred arterial tissues and mapping fluorescent protein localization. Subsequent measurements by confocal microscopy showed statistically significant differences in average fluorescent signal intensity between the control and transferred tissues. This result was corroborated by immunohistochemical staining. These preliminary results are encouraging evidence that the optical imaging method can be developed further to be performed non-invasively and in vivo in a clinical setting.**

***Keywords*- Optical imaging, green fluorescent protein (GFP), gene therapy, confocal microscopy, cardiovascular disease**

I. INTRODUCTION

Treatment of cardiovascular diseases presents a tremendous problem in healthcare. More than 60 million Americans suffer from some form of cardiovascular disease that will result in an estimated cost of $298B [1]. Among cardiovascular diseases, atherosclerosis is a leading cause of death [1]. Recently, gene therapy approaches to treating atherosclerosis have shown promise by preventing thrombosis and restenosis and by promoting angiogenesis [2-4]. The success of gene therapy is highly dependent on gene transfer efficiency and sufficient expression of the therapeutic gene [5]. Thus, there is a need to develop an imaging method that can precisely map and monitor the localization of gene expression *in vivo* in the vasculature.

An increasingly common marker in gene therapy is green fluorescent protein (GFP). Derived from the jellyfish *Aequorea Victoria*, GFP has been used to track gene expression and protein localization by fusing GFP to other proteins [5, 6]. The fluorescence emitted by GFP can be detected optically. However, applications for optical imaging have concentrated on fluorescence spectroscopy for diagnosis of neoplasia and for the discrimination of normal and atherosclerotic arterial tissue [7, 8]. Experiments to monitor gene expression with GFP and optical imaging have been performed on the Xenopus eye, tumors, and bronchus [9-12]. To our knowledge, optical imaging of GFP for the purpose of evaluating gene therapy in the cardiovascular system has not been explored.

Recently, members of our group have developed an optical imaging system that successfully detected GFP expression from vascular smooth muscle cells (SMCs) *in vitro* and arterial tissue *ex vivo* [13]. The optical imaging method described in this paper is based on that system, but we extend the concept such that imaging can be done *in vivo* and noninvasively. The overall goal is to develop a non-invasive optical imaging method for tracking vascular gene expression *in vivo*. The specific objectives include: (1) developing a noninvasive optical imaging system; (2) comparing the effectiveness of this system for detecting GFP and red fluorescent protein (RFP) expression; (3) validating the optical imaging results with confocal microscopy and immunohistochemistry; (4) performing Monte Carlo simulations based on optical tissue properties to enhance the capabilities of this system; and (5) evaluating the clinical feasibility of this imaging system to monitor vascular gene therapy.

II. METHODS AND MATERIALS

A. Gene-Vector Transfer

Two types of fluorescent protein genes, GFP and RFP genes, were used for comparison. GFP has a greater gene transfer efficiency due to its viral vector [4], but its emission spectra intersect many endogenous fluorophores, resulting in more background noise or autofluorescence [8]. RFP excitation and emission maxima have longer wavelengths, and it has been shown that photons of longer wavelengths penetrate more deeply into tissue [8].

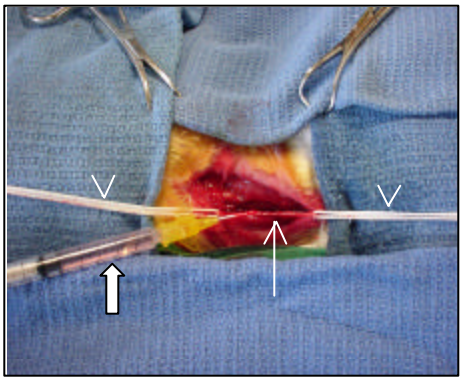

Fig. 1. Surgical-based gene delivery into the carotid artery (long arrow). After isolating the artery with two Sentinel loops (arrowheads), the RFP-vector solution (block arrow) was injected into the arterial lumen.

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The method by which the animal was prepared and the GFP vector delivered was adapted from previously developed protocol (Fig. 1) [13]. Using a third-generation lentivirus, we produced a GFP-lentiviral vector that was transferred for one hour into the right femoral artery of one New Zealand white rabbit, approximately 4 kg in weight. For RFP-plasmid transfection (20µg/mL, pDsRed-N1; Clontech Laboratories, Inc., CA), the right carotid arteries of two New Zealand white rabbits were transferred for two hours using the same surgical approach. The left femoral and carotid arteries were not transferred and served as controls. The animals were subsequently allowed to survive for 5 days to enable peak GFP expression and for 1.5 days for peak RFP expression.

B. In Vivo Optical Imaging

The optical imaging system is depicted in Fig. 2. The system included a charge coupled device (CCD) video camera (SensiCam, Cooke Corp., MI) that interfaced to an image-grabber board installed on a personal PC (Dimension 4100, Dell Computer Corp., TX). Imaging software included IPLab Scientific Image Processing v3.070 (Scanalytics Inc., VA) and NIH Image v1.62 (U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image). A series of lenses (Fujinon-TV, Fuji Photo Optical, Japan; Nikon AF Nikkor, Nikon, Japan) and emission bandpass filters (XF1072, Omega Optical Inc., VT; HQ545/30, Chroma Technology Corp., VT) were mounted onto the camera. The emission filters were selected to capture the specific fluorescence wavelengths of GFP or RFP. Broadband light from a 250W halogen light source (Schott KL2500 LCD, Schott Group, Germany) was filtered by excitation bandpass filters (XF3080, Omega Optical Inc., VT; HQ610/75, Chroma Technology Corp., VT) that selectively passed excitation wavelengths for GFP or RFP. A fiber-optic ring-light (Nevoscope, TransLite, TX) transmitted filtered excitation light onto the skin or the target vessel.

After peak expression of GFP or RFP, the transferred arteries were surgically exposed and isolated for optical imaging. The camera was placed directly onto the target vessel, and images were captured for various exposure times (1, 2, and 5 seconds). To visualize GFP or RFP, their respective filter sets (excitation and emission filters) were used. Upon completion of optical imaging, we euthanized the animals and harvested the control and target arteries. Both arterial specimens were cut into two equal-sized pieces: one for confocal microscopy and the other for immunohistochemical staining.

C. Confocal Microscopy

To assess the reliability of the technical development, we compared the results from optical imaging and confocal microscopy. A confocal microscope (Zeiss LSM 410; Zeiss Group, Germany) was used to image and measure average intensity from sections of fresh control and fresh target arterial tissue mounted onto slides. Excitation of GFP and RFP was achieved with an Ar laser (488 nm wavelength) and a green HeNe laser (543 nm), respectively. Filters were set to maximally capture the specific emission fluorescence spectra of GFP or RFP. Individual smooth muscle cells were visualized with a 40X lens (Zeiss C-Apo X40; Zeiss Group, Germany). For each of the control and target tissues, average intensity measurements were recorded for at least 27 randomly selected sites, with each site covering an area of 0.0256 mm^2. This process was repeated by several investigators to reduce individual subjective bias.

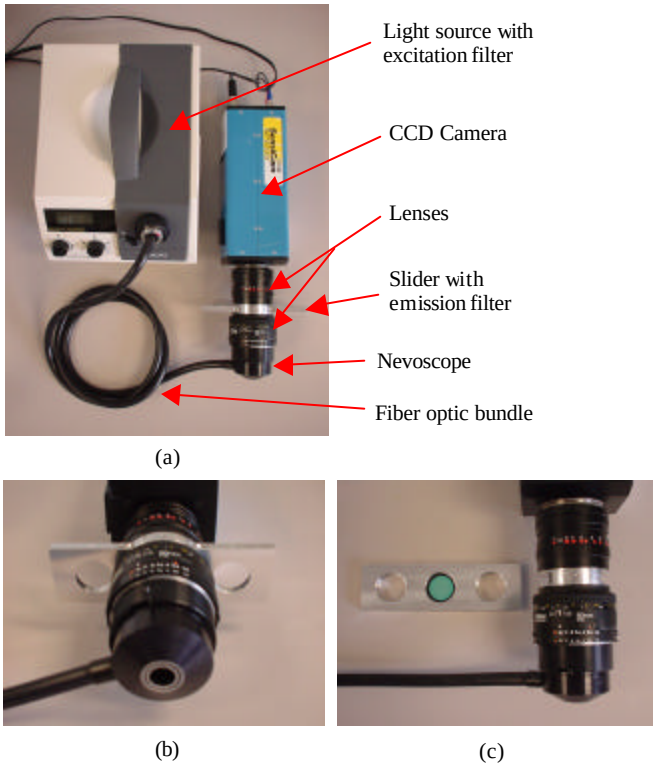


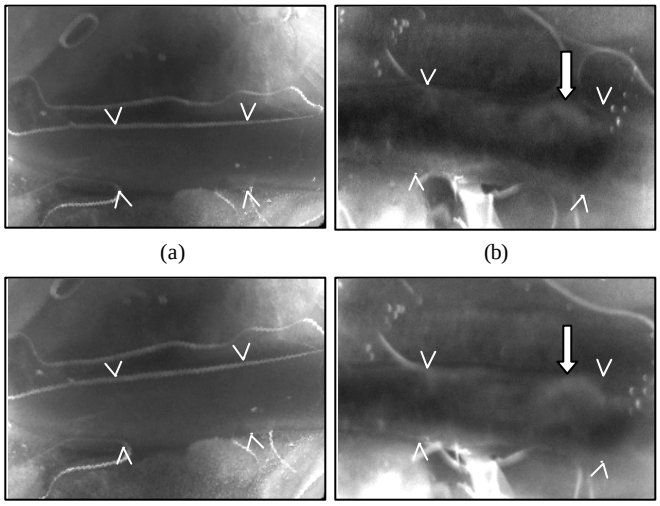
Fig. 2. Optical Imaging System: (a) light source, fiber bundle, and CCD camera system; (b) front view of fiber-optic ring-light (Nevoscope); (c) removed slider with emission filter (center slot). Note: PC not shown.

D. Immunohistochemistry

To confirm the success of the primary GFP/RFP-vector transfer, we performed immunohistochemical staining using specific monoclonal antibodies for GFP (Roche, IN) or RFP (Clontech Laboratories, Inc., CA). Verification by immunohistochemical analysis followed previously developed methods [13].

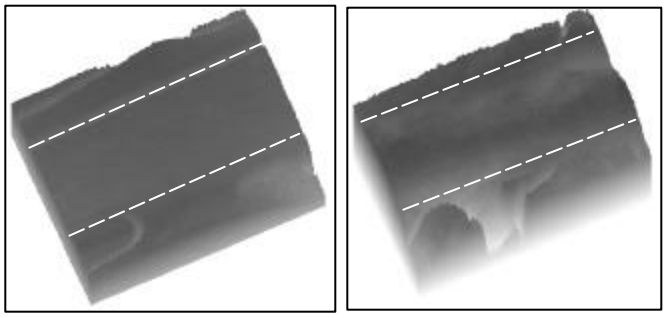
III. Results

Images of control and RFP-transfected carotid arteries captured by the optical imaging system are shown in Fig. 3. GFP images are not shown. The distinct area of brighter signal along the vessel wall, indicated by the block arrow, suggested that RFP was expressed and was emitting fluorescence. A comparison of the 2- and 5-second exposure images revealed that the area of brighter signal expanded as the exposure time increased and indicated that the area was representative of RFP fluorescence (Fig. 3(b)(d)). This direct relation between detected fluorescence and exposure time was expected because longer exposure times allowed the camera to collect more photons emitted by RFP. In contrast, the arterial wall of the control tissue did not show changes in intensity as the exposure time was increased (Fig.



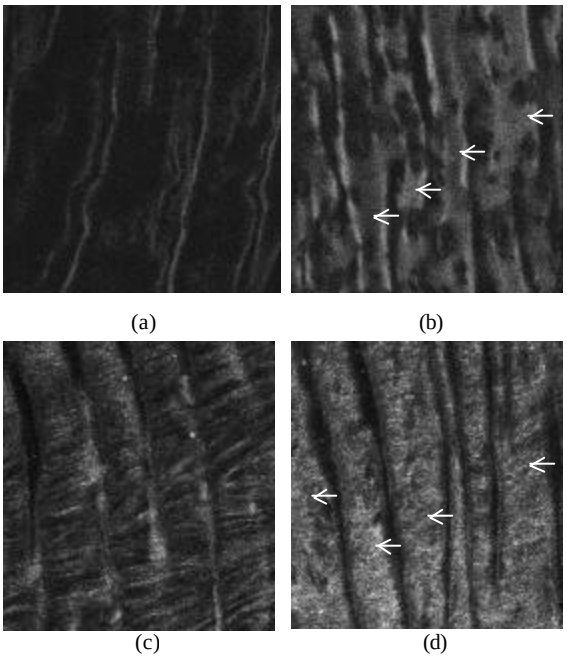
(a) (b) (c) (d)

Fig. 3. Direct-contact images of surgically-exposed carotid arteries (outlined by arrowheads) captured by optical imaging. Control artery imaged at (a) 2- and (c) 5-second exposures; RFP-transferred artery imaged at (b) 2- and (d) 5-second exposures. Note that the bright region of RFP fluorescence (indicated by block arrow) expanded with longer exposure time (b)(d). No such region was detected in controls (a)(c).



(a) (b)

Fig. 4. Surface plots of intensity across the width of a section of the vessel from Fig. 3(a)(b). Intensity was flat across the control artery (a), but heterogeneous for the target artery (b) due to contribution from RFP fluorescence. The dashed line was added to denote the vessel boundary.



(a) (b) (c) (d)

Fig. 5. Confocal images of the (a) control and (b) GFP-transferred femoral artery and of the (c) control and (d) RFP-transferred carotid artery. In (b) and (d), the GFP/RFP fluorescence delineates the smooth muscle cells (SMCs) of the target artery more clearly than the autofluoresence from SMCs of the control (a)(c). Arrows indicate areas of fluorescence emitted by GFP/RFP from the cytoplasm of SMCs. Note that the autofluorescence intensity is greater for the carotid artery than the femoral artery.

TABLE 1
t-TEST RESULTS FOR AVERAGE FLUORESCENCE SIGNAL INTENSITY MEASUREMENTS

	Average Intensity (SD) of Control Tissue	Average Intensity (SD) of Transferred Tissue	p-value (95% Confidence)
GFP[a] (N=27)	40.0 (4.2)	56.1 (6.3)	0.0000 (<0.05)
RFP_1[b] (N=27)	60.6 (11.8)	69.3 (12.1)	0.0002 (<0.05)
RFP_2[b] (N=30)	53.1 (10.0)	61.7 (12.0)	0.004 (<0.05)

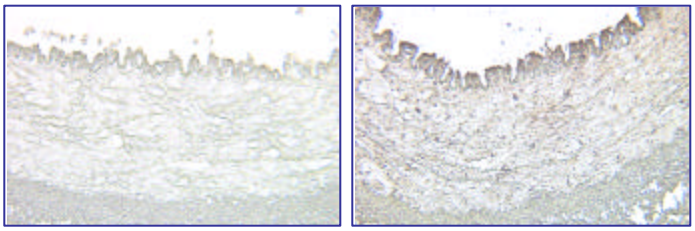
[a]Treatment site: femoral artery.
[b]Treatment site: carotid artery; subscripts denote trial number.

3(a)(c)). The intensity distribution for the control was homogeneous because there were no fluorophores present. The bright lines in Fig. 3 were artifacts from the reflection of the edges of saline fluid contacting the transparent film taped across the Nevoscope. Fig. 4 illustrates surface plots of intensity for sections of control and RFP-transferred arteries from Fig. 3(a)(b). The plot in Fig. 4(b) demonstrated that the intensity values peaked across the width of the target vessel whereas the plot for the control in Fig. 4(a) was flat. These dramatic differences confirmed the contribution of RFP fluorescence to overall intensity.

Confocal microscopy was able to verify the success of the gene transfer and protein expression. Fig. 5 illustrates the smooth muscle cells of the control and GFP/RFP-targeted arterial tissue. Confocal images of the control tissue in Fig. 5(a)(c) showed tissue autofluorescence. In contrast, the fiber morphology of smooth muscle cells were clearly delineated in both GFP- and RFP-transferred tissue in Fig. 5(b)(d). The bright areas between the cell membrane (indicated by arrows) represented fluorescence emitted from GFP or RFP. This result indicated that the SMCs were successfully transferred with the fluorophore gene and had actively expressed the protein. Average intensities measured by confocal microscopy for control and target arterial tissues were compared using a paired t-test (Intercooled Stata 6.0; Stat Corp, TX). The results shown in Table 1 revealed statistically significant differences between control and target tissues for both GFP and RFP trials. Moreover, results of the immunohistochemical staining for GFP in Fig. 6 indicated the presence of GFP in the arterial wall. Hence, the corroborative results from confocal microscopy and immunohistochemistry signified that the SMCs were expressing fluorophores which emitted fluorescence detected by optical imaging.

IV. DISCUSSION

The advent of gene therapy as a potential treatment for cardiovascular diseases has created a need for an imaging modality capable of detecting gene expression. Exogenous fluorophores such as GFP and RFP enable us to tag and localize therapeutic



(a) (b)

Fig. 6. Images of histological sections of (a) control and (b) immunohistochemically stained arterial tissue. Stained GFP forms brown-colored precipitates, resulting in a color change of the entire arterial wall from gray (a) to brown (b). 200X magnification.

proteins via optical imaging. However, the application of optical imaging for monitoring cardiovascular gene therapy has not been explored. Hence, the overall goal of this work is to develop a novel, noninvasive optical imaging method that can be implemented clinically to evaluate the success of vascular gene transfer and to monitor the progress of vascular gene expression over time.

Previous work on optical imaging of GFP from arterial tissue established the conceptual feasibility [13]. Based on this work, the system presented in this paper takes the concept one step closer to clinical application. To date, we have performed experiments to detect GFP- and RFP-fluorescence *in vivo* by directly contacting the vessel wall. The preliminary results obtained from this method illustrate that our proposed imaging method can discern the difference between normal and transfected tissue. Furthermore, the images show the areas where the fluorophores are located along the vessel wall. Thus, the optical imaging method is able to detect gene expression and map protein localization upon direct contact with the artery.

The next challenge is to optically image gene expression from near-surface vessels noninvasively and *in vivo*. To achieve this, we will use a Monte Carlo model to simulate the propagation of light transport in tissue based on tissue optical properties (e.g. absorption, scattering, and anisotropy) [14, 17]. Monte Carlo simulations have been used extensively to assess the effects of tissue optical properties, excitation beam profiles, and collection geometries on fluorescence [8, 15, 16]. Results from these simulations will provide insight into optimizing the design of the optical imaging device and method. For example, we can calculate the number of photons emitted from GFP or RFP and determine what fraction is transmitted through the tissue and detected by the camera. In addition, we are planning to use a Xenon lamp or laser as an alternative light source due to their greater radiant intensity at the desired excitation wavelengths. These characteristics will extract the maximum amount of fluorescence from GFP or RFP [8].

V. Conclusion

Further work is necessary to optimize and refine the optical imaging method presented here. We are currently working to develop a noninvasive, *in vivo* imaging method based on optical imaging of GFP from SMCs *in vitro*. We are confident that implementing the aforementioned strategies will help us accomplish our goal of creating a novel, noninvasive optical imaging method that be used clinically for monitoring cardiovascular gene therapy.

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